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Temperature-dependency of transformation and cell cycle length in human lymphocytes

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TEMPERATURE-DEPENDENCY OF TRANSFORMATION AND CELL CYCLE LENGTH IN HUMAN LYMPHOCYTES

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John Patrick McGee

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APPROVAL SHEET

This thesis is submitted in partial fulfillment of the requirements for the degree of

Master of Arts

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Approved, May 1981

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TABLE OF CONTENTS

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LIST OF TABLES

LIST OF FIGURES

ABSTRACT

In vitro mammalian cell cycle length is dependent on many conditions including incubation temperature. In general, the growth rate of cultured mammalian cells is highest at temperatures slightly above the normal physiological temperature of the animal of origin.

Phytohemagglutinin (PHA) stimulated human lymphocyte cultures were incubated for 72 hr at two degree intervals from 31° to 43°C. From the time of initiation, each culture was continuously labeled with 5-bromodeoxyuridine (BUdR). The growth rate of each culture was determined from metaphase labeling patterns. Fastest growth occurred at 39°C; growth at 37°, 35°, 41°, and 33° was progressively slower. No mitotic activity was seen at 31° and 43°C.

To determine the role of temperature-dependency of transformation in differential growth rates, six cultures were incubated at 35°, 37°, 39°, and 41°C. Fifteen hours after initiation, tritiated thymidine was introduced to one culture from each series. Three hours later, these cultures were terminated and tritiated thymidine was added to another culture from each series. After all were successively treated in this manner, autoradiographs were prepared and counts made of labeled cells. This 18 hr profile of transformation rates demonstrated that cells grown at 39°C transformed the fastest.

To determine the role of cell cycle length on the temperature-dependency of growth rate, lymphocyte cultures were incubated with PHA for 96 hr at 37°C, transferred to fresh medium with BUdR, and then incubated at 31°-43°C for an additional 72 hr. Analysis of growth rates revealed a broad peak at 37°-39°C. Again, no mitotic activity was observed at 43°. A low growth rate was seen in cells grown at 31°C.

The lengths of cell cycle phases were determined through serial labeling of lymphocyte cultures with BUdR. $M+G_1$ and G_2 were shortest at 39°C, although durations at 37°C were essentially similar. Durations of both in cells at 35° and 41°C were somewhat longer, but similar to one another. S phase was shortest at 35°C and increased with increasing incubation temperature.

It is suggested that 39°C should be the incubation temperature of choice for at least some further studies involving lymphocytes and that the maximal growth at 39°C may be a functional aspect of the lymphocyte's role in the immune response.

TEMPERATURE-DEPENDENCY OF TRANSFORMATION AND CELL CYCLE LENGTH IN HUMAN LYMPHOCYTES

INTRODUCTION

An animal cell *in vivo* exists in a complex and highly ordered environment. Its behavior is certainly controlled by its genome, but this behavioral control is defined in part by the extracellular environment. Because of this environmental definition, the *in vitro* growth and division of animal cells are rigidly controlled by culture conditions such as pH of the medium (Eagle, 1974), population density (Sanford, Earle, and Likely, 1948; Abercrombie and Heaysman, 1954; Eagle and Piez, 1962), nutrients supplied by the culture medium (Eagle, 1955), and incubation temperature.

That incubation temperature imposes control over the ability of cultured cells to grow and divide was recognized early in the development of successful cell culture techniques. Carrel (1912) described two phases of the *in vitro* existence of chick embryo fibroblasts; these he called "active life" and "latent life". "Active life" was defined as numerical growth with concomitant depletion of medium nutrients by cells maintained at 38°C. "Latent Life", on the other hand, was a non-proliferative phase exhibited by cells incubated at 0°C. Kodama (1930) demonstrated that chick embryo fibroblasts maintained at approximately 0°C for 1-2 days regain their proliferative ability when transferred to fresh medium at 39°C. Kodama also found that cells maintained up to 10-12 days at 25° and 30°C were able to resume active division upon transfer to fresh medium at 39°C. However, cells maintained at 25°C were better able to resume division than those maintained at 30°C.

Certainly, then, cellular growth and division are temperature-dependent. Implicit in the term "temperature-dependent" is the concept of an optimal temperature at which maximal growth and division occur. One might expect the optimal temperature for cell growth and division to approximate the normal body temperature of the animal of origin. This appears to be the case. Mammalian and avian cells in vitro thrive at temperatures near their characteristic physiological temperature (specific references to be discussed below). Cells from ectotherms, those vertebrates which rely on external sources of heat for body temperature regulation, exhibit maximal growth and division at temperatures approximating those of the preferred habitat. Whether normal body temperature is a behavioral or physiological response to cellular temperature optima or cell temperature optima are the evolutionary consequences of preferred habitat or physiological temperature control are points for speculation. In either case, cell temperature optima are indicative of the organism's physiological temperature.

One example of an optimal in vitro temperature paralleling the preferred thermal habitat of an ectotherm is that observed in a fibroblast-like cell line derived from gonadal tissue of the rainbow trout Salmo gairdneri (Plumb and Wolf, 1971). Peak cell growth occurs at 20°C with a doubling time of 2.0 days. Above and below 20°C growth rates diminish, with incubation at 30°C lethal for S. gairdneri cells. At 5°C, the cells remain viable but exhibit a mean doubling time of 13.8 days. This in vitro thermal profile fairly accurately matches the optimal habitat of the rainbow trout which thrives in waters not warmer than about 21°C (Eddy and Underhill, 1971).

Endotherms present a slightly more complex situation due to their

internal heat production. Whereas the body temperature of an ectotherm is generally equivalent to the temperature of its environment, the body temperature of an endotherm may well be higher than that of its environment, particularly in temperate and arctic species. In mammals, the skin has an *in situ* temperature of 32°-33°C (Rothman, 1954) as opposed to core temperatures generally in the range of 36°-39°C (Thestrup-Pedersen, 1972; Feldberg, 1975; Dinarello and Wolff, 1978). It is of interest to ask whether the optimal *in vitro* temperature of skin cells is lower than normal core temperature. Epidermal basal cells from neonatal mice will undergo stratification *in vitro* similar to *in vivo* epidermal development. When incubated at 37°C, stratified cultures will degenerate after 22-25 days (Fusening and Worst, 1975) whereas stratified cultures maintained at 32°-33°C exhibit an extended *in vitro* life span with no decrease in proliferative capacity by day 25 (Marcelo et al., 1978). However, human epidermal cells *in vitro* do not grow at 32°-33°C but exhibit optimal growth at 35°-36°C (Eisinger et al., 1979).

Another mammalian tissue which has an optimal temperature lower than the core temperature is testicular tissue (Bloom and Fawcett, 1975; Hickman et al., 1979). Spermatogenesis will not proceed at core temperature in many mammals. In these species, the testes will descend into the scrotum and thus will be maintained at a slightly lower temperature, one which permits spermatogenesis to proceed.

Other tissues, particularly those of the inner viscera, are maintained at the endotherm's core temperature. In light of above descriptions of temperature optima, it seems likely that these tissues would exhibit optimal *in vitro* growth at or near core temperature. This has

been found to be the case in a variety of mammalian tissues in culture.

Mice have a normal core temperature of 36.5°C (Schmidt-Nielsen, 1964). Cultured murine leukemic cells have been shown to have a shorter generation time when incubated at 37°C than when incubated at 28°, 31°, 32°, 34°, or 40°C (Watanabe and Okada, 1967). The generation time observed in cells maintained at 40°C was only slightly longer than that observed in cells at 37°C. However at 34°C, the generation time was nearly twice that observed in cells at 37°C. It may well be that the shortest generation time would actually occur at a temperature between 37° and 40°C.

Human cells appear to follow this pattern (Sisken, Morasca, and Kibby, 1965). Amniotic cells incubated at 39°C exhibit a slightly shorter intermitotic time than do cells at 37°C. However, at 38°C the intermitotic time is longer than in cells at 37°or 39°C. At temperatures above and below the 37°-39°C range, intermitotic times increase sharply. Somewhat similar results have been observed with HeLa cells (Rao and Engelberg, 1965). In HeLa, generation times at 38°C were shorter than at 37°C; however, no determination of generation time was performed on cells grown at 39°C. Again, generation times of cells incubated at temperatures above and below the optimum increased sharply.

Thus, mammalian cells that normally exist at core temperature _in vivo_ exhibit optimal _in vitro_ proliferation at incubation temperatures 1-2 degrees higher than core temperature. At temperatures above and below the observed optimum, the _in vitro_ cell cycle will be prolonged.

There is conflicting evidence on which phase of the cell cycle is more subject to length perturbation by temperature. For conditions other than temperature, G_1 is regarded as the most variable phase of

the cell cycle (Defendi and Manson, 1963). Environmental factors such as suboptimal pH prolong G_1 as does contact inhibition in non-malignant monolayer cultures (Sisken, 1963; Todaro, Lazar, and Green, 1965). Incubation temperature, on the other hand, may affect all phases of the cell cycle and in different ways characteristic of each phase. In human amnion cells, G_1 showed the highest thermosensitivity of any phase, G_2 was the least thermosensitive, and S was intermediate (Sisken et al., 1965). In mouse leukemic cells, G_1 and S were most prolonged by suboptimal temperature (Watanabe and Okada, 1967). In HeLa however, G_1, S, and G_2 durations were affected similarly and proportionally by supra- and suboptimal temperatures (Rao and Engelberg, 1965). A disproportionate increase in mitotic duration was observed in HeLa cells maintained at suboptimal temperatures. This increase in the duration of M was so profound that at temperatures ranging from 26°-33°C, mitotic indices as high as 0.44 were noted, indicating that 44% of the cells scored were in metaphase.

Lymphocytes <u>in vitro</u> have an additional cycle phase - G_0. In terms of <u>in vitro</u> lymphocyte kinetics, transformation is the entry of the quiescent, or G_0, lymphocyte into G_1 of the division cycle. (For a review of morphological and biochemical events in transformation, see Naspitz and Richter, 1968). As with other phases of the cell cycle, transformation is thermosensitive. When pulse-labeled with ^{3}H-thymidine (^{3}H-TdR) for several hours before fixation at 1, 2, or 3 days, phytohemagglutinin (PHA)-stimulated human lymphocytes incubated at 39°C incorporate label earlier than do those incubated at 37°C (Ashman, Gomez-Barreto, and Nahmias, 1976; Ashman and Nahmias, 1977; Ashman and Nahmias, 1978). Similar results are observed when Concanavalin A (Con A)

is employed as the mitogen (Ashman and Nahmias, 1978).

One may argue that differences seen in rates of transformation are actually reflections of temperature-dependent differences in proliferation rates. Through sequential addition of the saccharide methyl-α-D-mannopyranoside (MMP), which inhibits Con A binding, to sets of Con A-stimulated lymphocytes at 37° and 39°C, Ashman and Nahmias (1978) obtained similar profiles for ^{3}H-TdR uptake between culture sets. On this basis, they concluded that incubation at 39°C does not increase the rate of transformation. However, figure 3 of Ashman and Nahmias clearly indicates that at each MMP addition time, more label was being incorporated at 39° than at 37°C. Since label incorporation was measured by liquid scintillation, it is not possible to ascertain whether more cells were synthesizing DNA or more label was incorporated per cell. In addition, samples were not measured until 3 days after culture initiation. If more cells are synthesizing DNA, their presence may be due either to increased numbers of cells being transformed, higher proliferation rates, or both. Whether incubation temperature actually influences the number of cells entering the mitotic cycle has yet to be adequately ascertained.

The purpose of the present study is two-fold. By knowing the effects of temperature on the cell cycle, one may know the optimal thermal environment for future in vitro studies. Attempts to determine optimal culture temperatures for non-human lymphocytes have been made (Thestrup-Pedersen, 1972; Brucher et al., 1973; Schneider and Goldman, 1974). These studies have shown that these cells have temperature optima other than the traditional 37°C. It is of interest to determine whether 37°C is the optimal temperature for human lymphocytes in vitro.

The second purpose of this study is to attempt to relate *in vitro* events with *in vivo* occurrences. Fever is one of the well-known responses of the vertebrate body to infection (Dinarello and Wolff, 1978; Kluger, 1978). What role does increased temperature have in the immune response? These questions will be dealt with in light of the results of this work.

In any study of cell division kinetics, a method for tracing the passage of cells through the division cycle must be employed. 5-bromodeoxyuridine (BUdR) is used extensively as a label in this study and, for this reason, a brief description of BUdR incorporation and labeling is appropriate.

BUdR is an analog of thymidine and will be incorporated into DNA at sites normally occupied by thymidine. Assuming uninemy and semiconserative replication, after one replication period in the presence of BUdR, each chromatid contains one BUdR-substituted strand of DNA and one non-substituted strand (unifilar substitution). After two replications in the presence of BUdR, one chromatid will contain a unifilarly substituted double helix and its sister chromatid will contain BUdR in both strands (bifilar substitution). Subsequent replication periods in the presence of label will lead to further bifilar substitution of the cell's genome. However, the original non-substituted strand will remain non-substituted, disregarding, of course, the phenomenon of sister chromatid exchange.

After two replication periods in the presence of BUdR, sister chromatids may be differentially stained by one of several methods. The method used in this study is an adaptation of the fluorescent-plus-Giemsa method introduced by Perry and Wolff (1974; see also Weber,

1979). After treatment and staining, unifilarly substituted chromatids will stain darkly and bifilarly substituted chromatids will be lightly stained. The reason such treatment produces sister chromatid differentiation is not clear. BUdR incorporation causes "differential spiralization" of chromosomes (Zakharov and Egolina, 1972), quenching of fluorescence of various dyes (Latt, 1973; Perry and Wolff, 1974; Kato, 1974), and the subsequent staining procedure causes loss of DNA (Webber, Brasch, and Smyth, 1981).

Because unifilar and bifilar substitution leads to differential staining of chromatids, the number of DNA synthetic periods a cell has undergone may be determined based on the number of light and dark staining chromatids present in a metaphase spread (Tice, Schneider, and Rary, 1976). First and second generation metaphases are readily identifiable; first generation metaphase chromosomes are uniformly stained whereas second generation metaphase chromosomes have one light and one dark chromatid. Subsequent generation metaphases, while not as readily identifiable as first and second generation metaphases, are nevertheless categorizable. Separation of chromatids at mitosis is a randon event in the sense that chromatids containing unsubstituted polynucleotide strands will assort independently into the two daughter cells (Heddle et al., 1967, Tice et al., 1976). Because chromatid assortment is random, third and subsequent generation metaphases may be recognized on the basis of the number of darkly stained chromatids present in the spread (Tice et al., 1976).

Cultures of non-synchronously dividing cells contain, by definition, cells in all stages of the cell cycle. When BUdR is added to a non-synchronous culture, cells in S phase will begin label incorpora-

tion within several minutes (Walters, Tobey, and Ratliff, 1973; Craig-Holmes and Shaw, 1976), whereas cells in other phases will obviously not incorporate the label until they enter S. Cells which were synthesizing DNA, i.e., were in S, at the time of BUdR addition may be recognized by the discontinuous staining pattern of their metaphase chromosomes. Chromatids will exhibit alternating light and dark regions, the distribution and extent of these regions depending on how far the cell had advanced into the S phase at the time of label addition (Grzeschik, Kim, and Johannsmann, 1975; Tice et al., 1976; Hoo and Parslow, 1979).

GENERAL MATERIALS AND METHODS

Lymphocytes for each experiment were obtained from heparinized venous blood from the same male donor. Each experiment was performed using blood drawn at one time; i.e., cultures in each experiment were grown simultaneously. For each culture, 0.4 ml of plasma containing lymphocytes and some erythrocytes was added to 4.5 ml medium composed of Ham's F10 (GIBCO) supplemented by 20% fetal calf serum (GIBCO or Flow Laboratories), 1% penicillin-streptomycin (GIBCO), and 90 μg/ml PHA (Wellcome). Cells were grown in 25 cm^2 plastic culture flasks (Falcon) kept tightly capped during the period of incubation.

All experiments involved simultaneous cell cultures incubated at various temperatures. Each temperature was maintained in a separate incubator and was monitored with a thermometer calibrated to a specific temperature with a reference thermometer. Calibration was performed using a tray of water heated by a slide warmer to the desired temperature. The water was kept in constant circulation throughout the tray to eliminate local temperature differences within the tray. When water temperature reached the calibration temperature, as indicated by the reference thermometer, the level of mercury was marked on the thermometer being calibrated.

Cultures to be terminated were first treated for 10 min with 0.075 M KCl. After centrifugation and removal of the KCl, cells were fixed in 3:1 (v/v) methanol:glacial acetic acid. Cells were treated

with two 10 min changes of fixative and were stored in a third fixative change. Fixed cells were dropped onto clean wet slides for examination.

BUdR (Sigma) was used to label cells in four of the five experiments. In each culture, the final BUdR concentration was 20 μg/ml culture medium. After termination, air-dried slides were stained for 60 min in a 20 μg/ml aqueous solution of Hoechst 33258 (American Hoechst Corp.). The slides were then placed in an enameled pan and covered with 2X SSC (0.3 M NaCl + 0.03 M Na-citrate, pH 7.0). They were then exposed for 40 min with a 15 watt ultraviolet germicidal lamp (General Electric) at a distance of approximately 25 cm. After UV-irradiation, more 2X SSC was added until the slides were covered to a depth of about 6 mm. The slides were then heated until the salt solution reached 70°C. Once achieved, this temperature was maintained for 15 min. Slides were then rinsed in hot tap water and stained. The stain used was an aqueous Giemsa composed of 1% Azure B in 0.05 M NaH_2PO_4 pH 6.8 and 1% Eosin Y in the same buffer. The final staining solution was a 5:2:30 mixture (Azure B:Eosin Y:buffer). (For more details on sister chromatid differentiation, see Appendix C in Weber, 1979).

It has been suggested that BUdR concentrations above approximately 11 μg/ml culture medium inhibit cellular kinetics (Tice et al., 1976). The concentration of BUdR used in each experiment in the present study was 20 μg/ml culture medium. This concentration of BUdR affords consistent differentiation of sister chromatids with high contrast between unifilarly and bifilarly substituted chromatids. If there has been an inhibitory effect because of BUdR concentration, each sample would most likely be affected to the same extent.

EXPERIMENT 1 - OVERALL GROWTH KINETICS

MATERIALS AND METHODS

To establish the temperature-dependence of overall lymphocyte growth and division kinetics, cultures of PHA-stimulated lymphocytes were incubated for 72 hr at two degree intervals from 31° to 43°C. BUdR was added to each culture at the time of its initiation and was present in the medium until termination. Colcemid was present for the last 2 hr of culture.

After termination and sister chromatid differentiation, 100 metaphases from each culture were scored for the number of divisions undergone and a growth rate index (GRI) was calculated for each culture. The GRI is defined as the sum of the number of cells that underwent a certain number of divisions, multiplied by that number of divisions for all cells scored in a sample. This number is then divided by the number of cells in the sample.

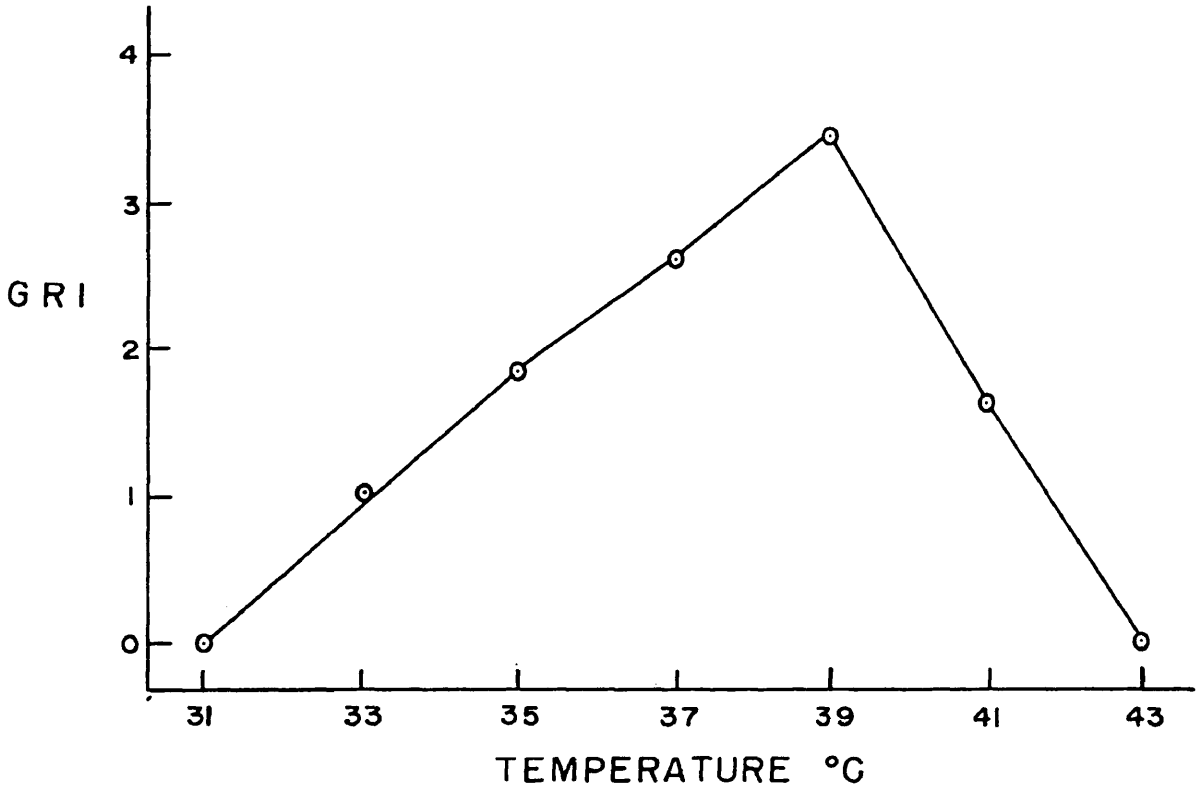
RESULTS

Calculated GRI values are shown in Fig. 1 plotted against the temperatures at which they were observed. The curve shows that the average number of lymphocyte divisions is influenced by incubation temperature. The peak GRI occurs at 39°C, indicating that cells maintained at 39°C underwent more divisions than did cells maintained at other temperatures. Temperatures above and below 39°C led to sharp decreases in GRI values.

No mitotic figures were seen in cells incubated at either 31° or 43°C. These temperatures may be non-permissive for either division or transformation, but are not necessarily lethal to the cells.

Cultures of stimulated lymphocytes contain populations of cells varying in their proliferative history (Crossen and Morgan, 1979). Cells having undergone three and four divisions comprise the majority of the samples at 37° and 39°C respectively. However, some third generation metaphases are seen at supra- and suboptimal temperatures, albeit at very low frequencies (Table 2).

Fig. 1. Experiment 1 results. Each point represents the GRI value calculated from 100 metaphases from each culture.


GRI
4
3
2
1
0
31
33
35
37
39
41
43
TEMPERATURE °C

EXPERIMENT 2 - THE TIME-COURSE OF PHA TRANSFORMATION

MATERIALS AND METHODS

Cells to be radioactively labeled were treated with ^{3}H-TdR (specific activity 20.0 Ci/mmol) (New England Nuclear) to a final activity of 0.2 μCi/ml culture medium. After termination, air-dried slides were prepared for autoradiography. Slides were dipped in Kodak NTB2 Nuclear Track Emulsion and stored at 4°C for one week. The slides were then developed and stained using a modification of Richardson's stain (Richardson, Jarett, and Finke, 1960). In the modified stain, Azure B was used rather than Azur II. After staining, the slides were dehydrated and mounted with Kleermount.

Six cultures of lymphocytes were incubated at each of four temperatures: 35°, 37°, 39°, and 41°C. Fifteen hours after initiation of the cultures, ^{3}H-TdR was added to one culture at each temperature. Three hours later, these four cultures were terminated and ^{3}H-TdR was added to another culture from each series. All cultures were successively treated in this manner. Autoradiographs were prepared and 1000 cells, both interphase and metaphase, from each culture were scored for radio-labeling. A cell was considered radio-labeled if at least 25 grains were present in the emulsion directly above the cell. The percentage of labeled cells for each culture was calculated.

RESULTS

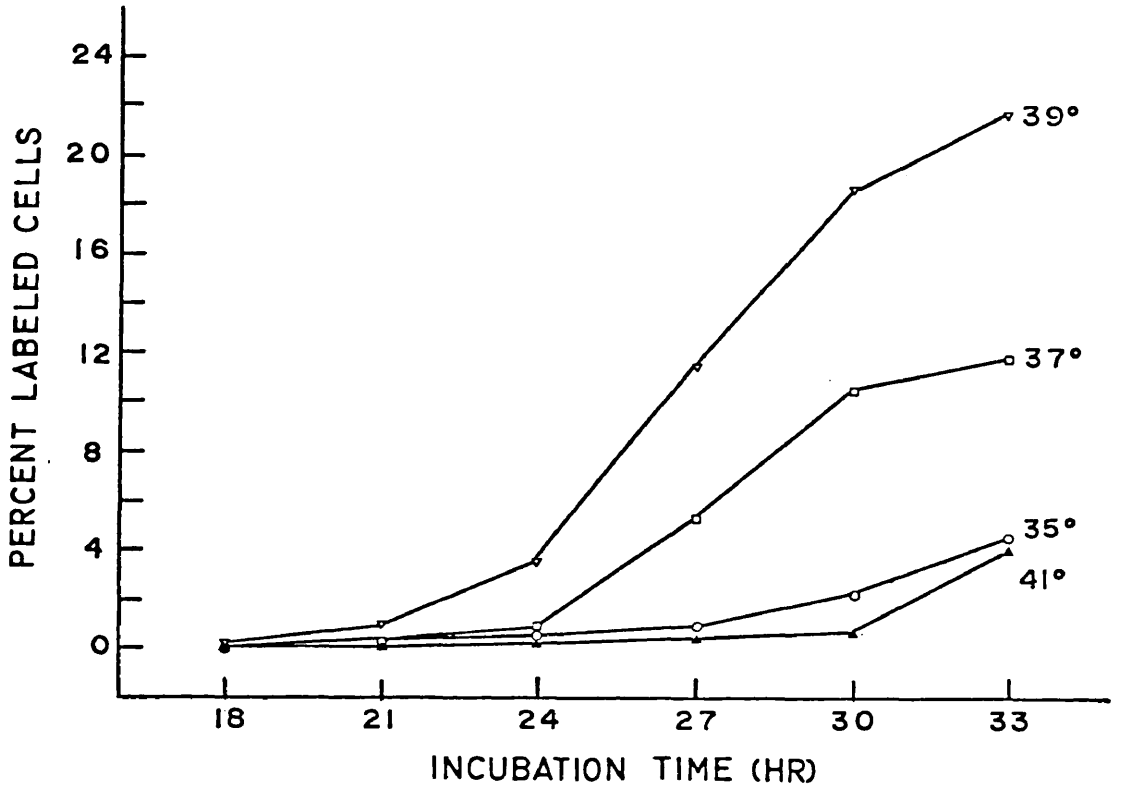
The temperature-dependence of growth observed in Experiment 1 may

be due either to a shorter cell cycle at or near the optimal temperature, an enhanced rate of transformation, or a combination of both. Previous investigations of the temperature-dependence of transformation have not adequately defined possible rate differences due to temperature. Here, by serial ^{3}H-TdR labeling and fixation of a set of cultures, an 18 hr profile of transformation rate at each of four experimental temperatures was obtained.

Fig. 2 shows the percentage of labeled cells plotted against the time from culture initiation to fixation. At incubation times of 27 to 33 hr, approximately twice as many cells at 39°C were synthesizing DNA compared to cells at 37°C. The transformation rates at 35° and 41°C were similar to one another and much lower than rates at 37° and 39°C.

The different rates observed are not due to differences in proliferative ability as Ashman and Nahmias (1978) have suggested. Among all samples counted, only two metaphases were seen, one each at 37° and 39°C, both at 33 hr. If proliferation rate, rather than transformation rate, differences were responsible for differences in number of labeled cells, then many more dividing cells would have been present. Since only two mitotic figures were observed, it seems reasonable to conclude that higher numbers of labeled cells are not the product of increased cell division and that transformation is a temperature-dependent process with the peak in vitro rate at 39°C.

Fig. 2. Experiment 2 results. The abscissa represents the total time of incubation for one culture from each temperature series. The cultures incubated for the times indicated contained tritiated thymidine (1.0 μCi) for 3 hr immediately prior to termination.


PERCENT LABELED CELLS
24
20
16
12
8
4
0
18
21
24
27
30
33
INCUBATION TIME (HR)
39°
37°
35°
41°

EXPERIMENT 3 - THE TIME-COURSE OF CELL DIVISION FOLLOWING TRANSFORMATION

MATERIALS AND METHODS

Seven cultures were initiated at 37°C with PHA but without label. After 96 hr at 37°C, the cells were then transferred to fresh medium containing BUdR but no PHA. Each culture was maintained at a different temperature over the range of 31°-43°C (at two degree intervals) for an additional 72 hr. Colcemid was present during the last hour of incubation. After termination and sister chromatid differentiation, 100 metaphases from each culture were scored for the number of divisions in the presence of BUdR, and a GRI for each temperature was calculated.

RESULTS

Transformation rate differences undoubtedly contribute to the temperature-dependence of overall lymphocyte kinetics; however, the division cycle may also be temperature-dependent, as it is in other mammalian cells. By preincubation of cultures at 37°C for 96 hr with PHA, each culture contained an equivalent pool of dividing lymphocytes at the beginning of the incubation period at the experimental temperature. Consequently, any differences in growth rate observed over the temperature range may be attributed to differences in cell cycle duration.

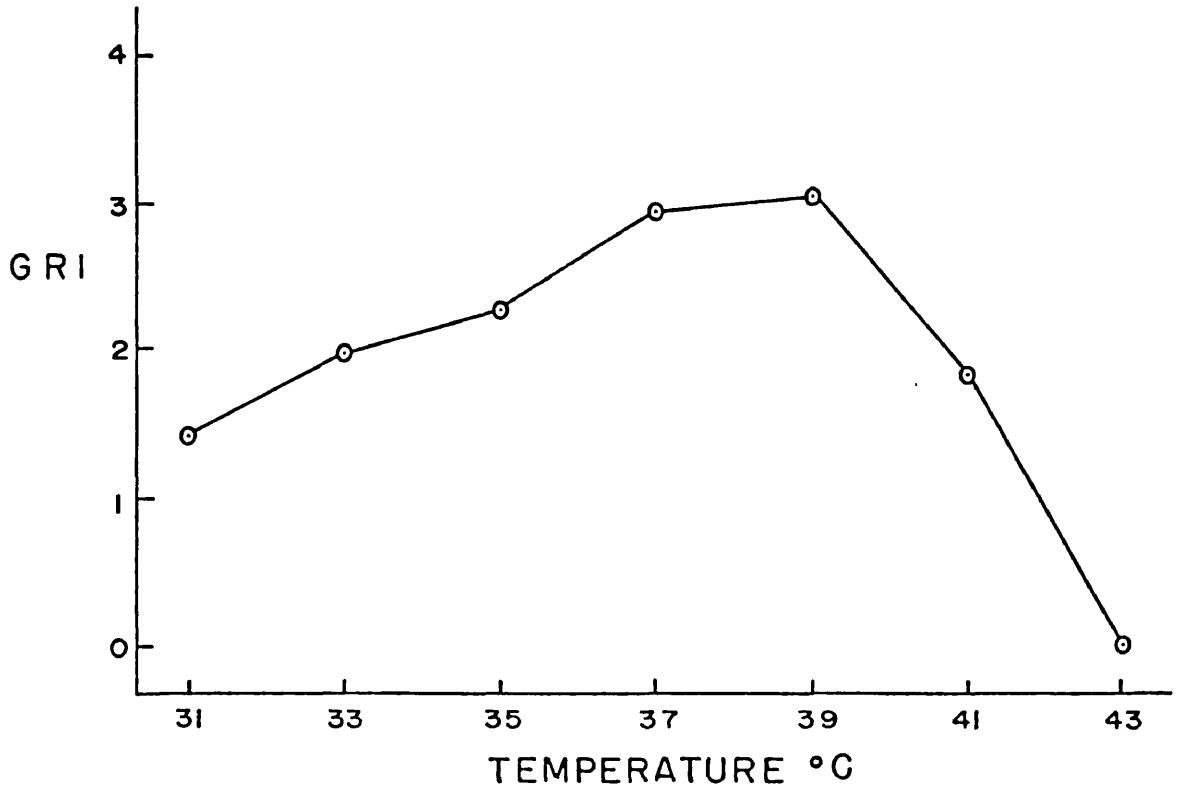
As in Experiment 1, GRI values are shown plotted against incubation temperature (Fig. 3). Again, there appears to be a temperature effect. There is, however, no distinct peak GRI. Indices at 37° and

39°C are 2.94 and 3.04, respectively. Apparently enhanced overall lymphocyte growth, as seen in the 39°C culture in Experiment 1 must have been due to the higher rate of transformation at 39°C with little or no difference in division cycle times between cells at 37° and those at 39°C.

No mitotic activity was observed in cells incubated at 43°C. It is possible that incubation at 43°C is not lethal, but merely non-permissive for mitosis. Zeuthen (1972) noted that in Psammechinus eggs, incubation at supraoptimal temperatures will cause disruption of the mitotic apparatus yet will allow the cell to continue non-proliferative growth with enlargement of cell volume. A similar phenomenon may exist for human lymphocytes at 43°C.

Mitotic figures were noted in cells grown at 31°C whereas none were evident in the 31°C culture in Experiment 1. Apparently transformation is inhibited at 31°C, but cell division may proceed, albeit at a low rate.

Fig. 3. Experiment 3 results. Each point represents the GRI value calculated from 100 metaphases from each culture.


GRI
4
3
2
1
0
31
33
35
37
39
41
43
TEMPERATURE °C

EXPERIMENT 4 - THE DURATION OF G_2

MATERIALS AND METHODS

Seven cultures of lymphocytes were initiated at each of four temperatures: 35°, 37°, 39°, and 41°C. Sixty-five hours after initiation, BUdR was added to one culture at each temperature. After 1 hr, BUdR was added to another culture from each series. This sequential addition of BUdR was continued until all cultures were labeled. All cultures were terminated 1 hr after addition of BUdR to the last culture from each series and addition of Colcemid to all cultures.

After termination and sister chromatid differentiation, 100 metaphases from each culture were scored. Cells which had been synthesizing DNA at the time of label addition were prominent due to the presence of replication bands on the chromosomes. Cells that had passed into G_2 prior to label addition exhibited no such bands; instead these chromosomes were uniformly stained.

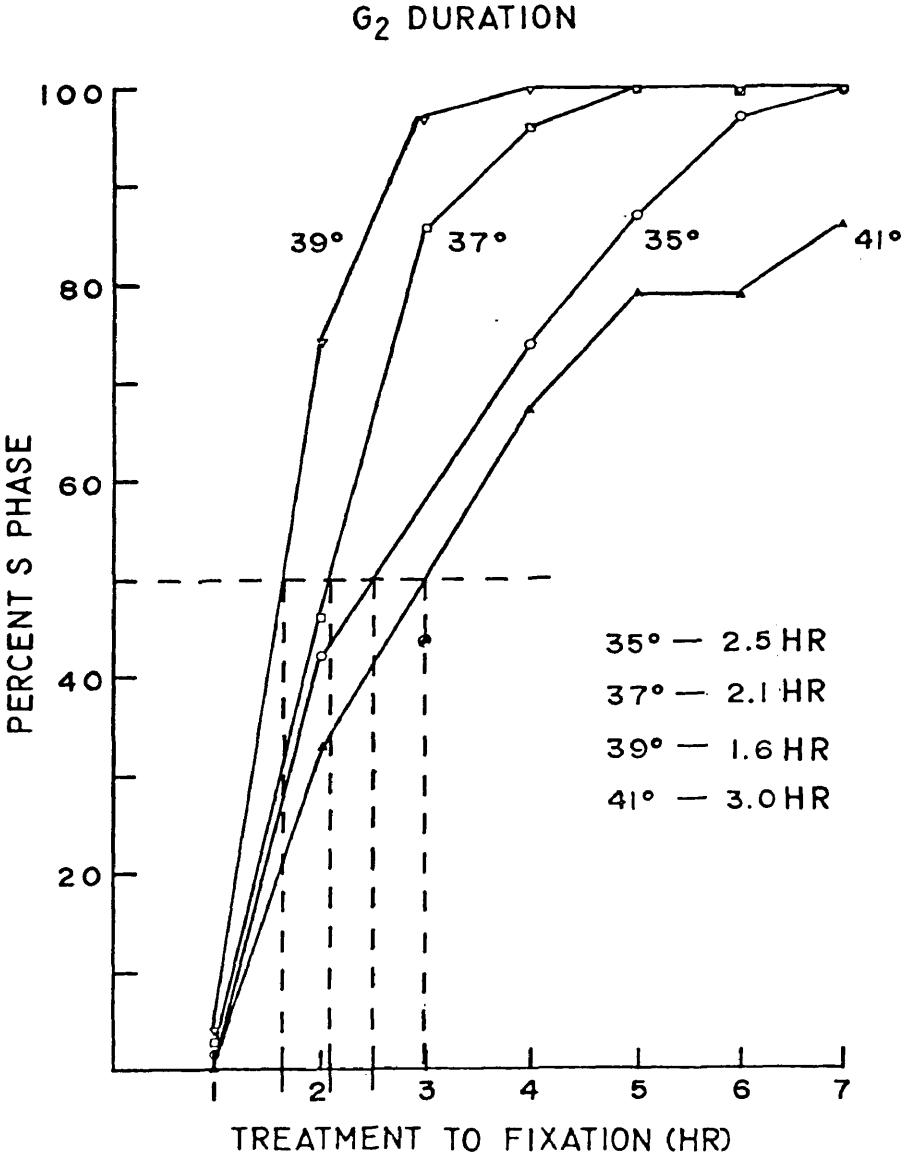
The percentage of cells scored as S phase from each sample was plotted against the length of time BUdR was present in the culture. The 50% level was chosen as the point of transition from S to G_2 of the sample population.

RESULTS

As shown in Fig. 4, the length of time a cell remains in G_2 appears to be dependent on temperature. Cells at 39°C had the shortest average G_2 time, 1.6 hr; G_2 times at 37°, 35°, and 41°C were progressively longer.

Cells incubated at 39°C also exhibited the least variability in G_2 length. Phase length variability was greatest at 41°C; at this temperature, 14% of the metaphases found 6 hr after BUdR addition had completed their last S phase prior to that time, even though the calculated average duration of G_2 was 3.0 hr. Such increased variability may have contributed to the anomalous counts at 3 hr for cultures at both 35° and 41°C.

Fig. 4. Experiment 4 results. The abscissa represents the time from addition of BUdR to termination of cells. Each point is the percentage of cells in S phase at the time of BUdR addition, in a sample of 100 metaphases from each culture. There were anomalous counts at 3 hr from cultures incubated at 35° and 41°C, probably due to variation inherent in populations of lymphocytes *in vitro*.


G_2 DURATION
100
80
60
40
20
PERCENT S PHASE
39°
37°
35°
41°
35° — 2.5 HR
37° — 2.1 HR
39° — 1.6 HR
41° — 3.0 HR
1
2
3
4
5
6
7
TREATMENT TO FIXATION (HR)

EXPERIMENT 5 - THE DURATIONS OF M+G_1 AND OF S

MATERIALS AND METHODS

Eight cultures were initiated at each of four temperatures: 35°, 37°, 39°, and 41°C. After 36 hr, BUdR was added to one culture from each series. Three hours later, BUdR was added to another culture in each series. All cultures were labeled at 3 hr intervals except the final culture in each series which was labeled 2 hr after the previous addition. All cultures were terminated 6 hr after the addition of label to the last culture in each series and after incubation with Colcemid for 1 hr.

As before, 100 metaphases from each culture were scored according to the pattern of label. Spreads that were uniformly stained were, at the time of label addition, in either G_2, mitosis, or the G_1 preceding the final S phase before termination. Spreads exhibiting replication bands were in the final S phase before termination. The percentages of metaphases which had been cells in these two categories were plotted against the time of incubation with label (Figs. 5-8). The point where each curve crossed the 50% level was taken as the phase transition time.

There were cells in several of the samples, especially in the 37° and 39°C cultures, which underwent two S phases in the presence of BUdR. These cells were not included in Figs. 5-8.

RESULTS

The percentages of the two categories of metaphase spreads, those stained uniformly and those with replication bands, are shown in Figs. 5-8 plotted against time of incubation with label. The plot of the percentage of uniformly stained spreads is designated G_2+M+G_1; the plot of the percentage of spreads with replication bands is designated S+G_2. The previously determined G_2 time was subtracted from each phase transition time to yield average M+G_1 and S durations. Some cells scored as G_2+M+G_1 at each temperature may not have undergone a previous division due to the presence of variable subpopulations inherent in lymphocyte cultures. For this reason, additional G_1 values in Table 1 are presented in parentheses; these values have not had the respective G_2 values subtracted.

As shown in Table 1, M+G_1 times appear to be temperature-dependent. The shortest duration is seen at 39°C; however, there is hardly any difference between this time and that seen at 37°C. The M+G_1 durations observed at 35° and 41°C are also similar to each other. Between the two temperature classes, however, there is a difference of approximately 4 hr.

The duration of the S phase presents a different pattern. The shortest S phase was observed in cells grown at 35°C (Table 1); S phase increases in length as incubation temperature increases. A similar increase in the duration of S with temperature was noted by Schneider and Goldman (1974) in lymphocytes from the opossum Didelphis virginiana.

Table 1 also includes, in parentheses, durations of S phase which have had 0.5 hr subtracted to compensate for the average time cells may have been arrested in metaphase by Colcemid.

Fig. 5. Experiment 5 results, 35°C. The abscissa represents the time from addition of BUdR to termination of cells. The curve designated as $S+G_2$ is of the percentage of cells in S phase at the time of label addition. The curve designated as G_2+M+G_1 is of the percentage of cells in either of those phases at the time of label addition. These cells, scored as uniformly stained chromosomes, also had to traverse S, G_2, and mitosis to metaphase. However, the segment of this curve between the two 50% levels represents only the time of G_2+M+G_1. Cultures undergoing early labeling also contained some cells which had gone through two replications in the presence of BUdR; these have not been represented on the graph, but the percentages of these cells in the counts are presented in Appendix A. The preceding descriptions also apply to the other graphs of the results of Experiment 5.

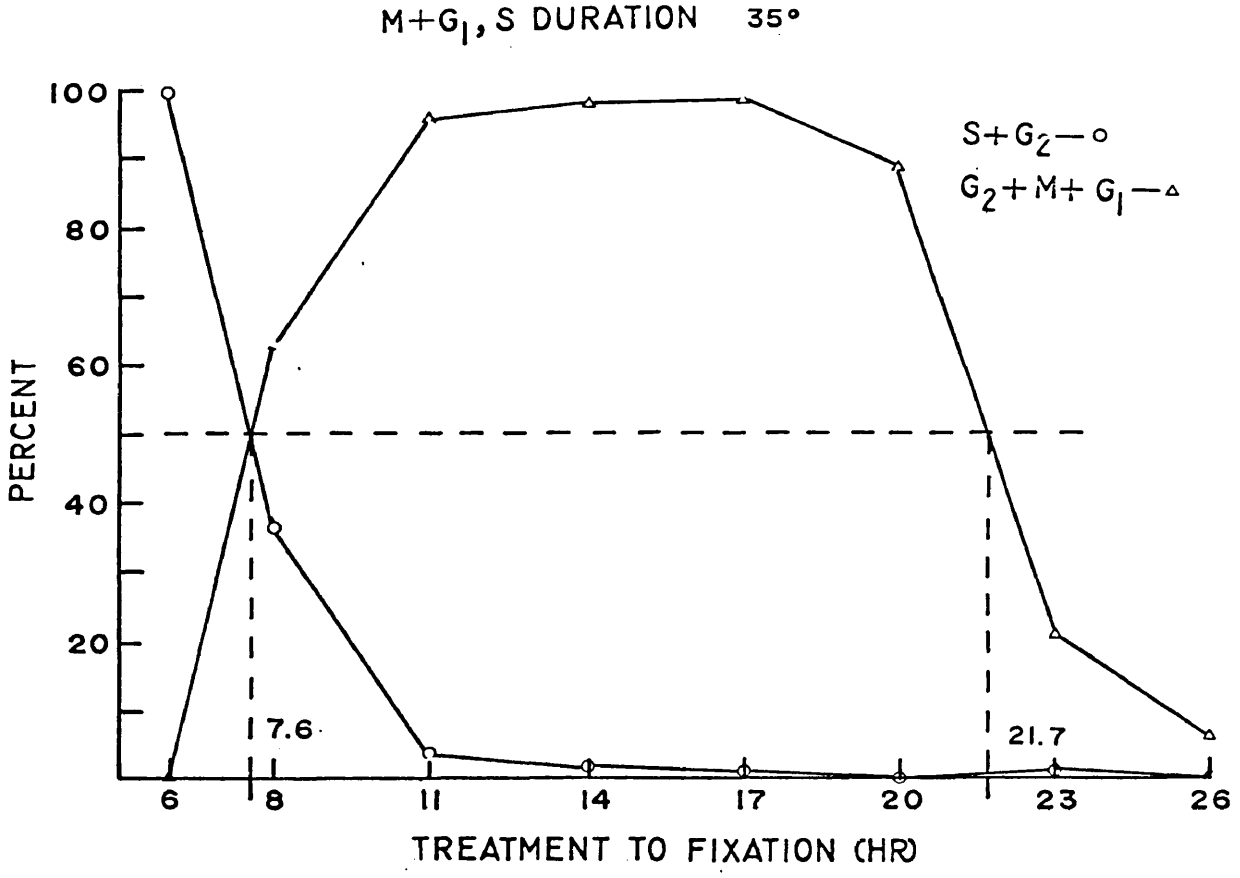

M+G1, S DURATION 35°
100
80
60
40
20
PERCENT
S+G2—o
G2+M+G1—Δ
7.6
21.7
6
8
11
14
17
20
23
26
TREATMENT TO FIXATION (HR)

Fig. 6. Experiment 5 results, 37°C. Percentages of 6 hr labeled cells are not included here, but are listed in Appendix A.

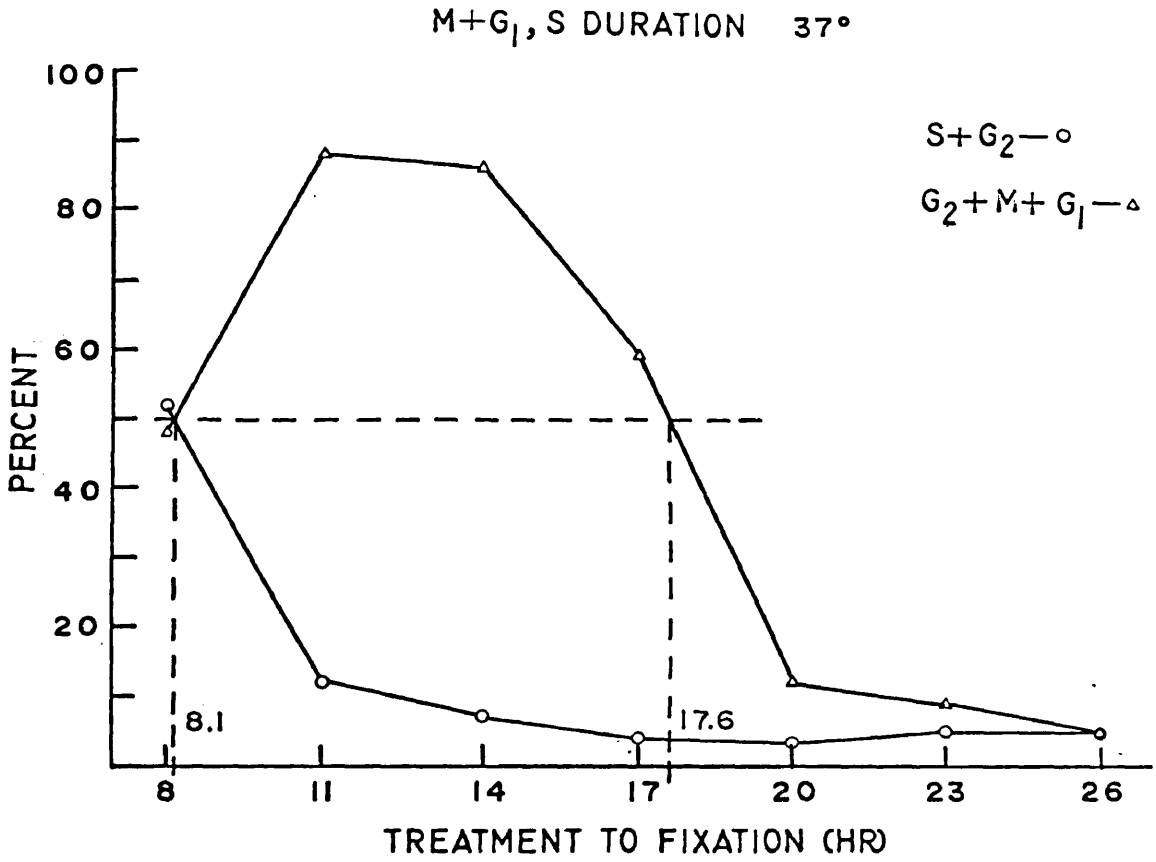

M+G1, S DURATION 37°
S+G2—o
G2+M+G1—Δ
PERCENT
100
80
60
40
20
8.1
17.6
8
11
14
17
20
23
26
TREATMENT TO FIXATION (HR)

Fig. 7. Experiment 5 results, 39°C.

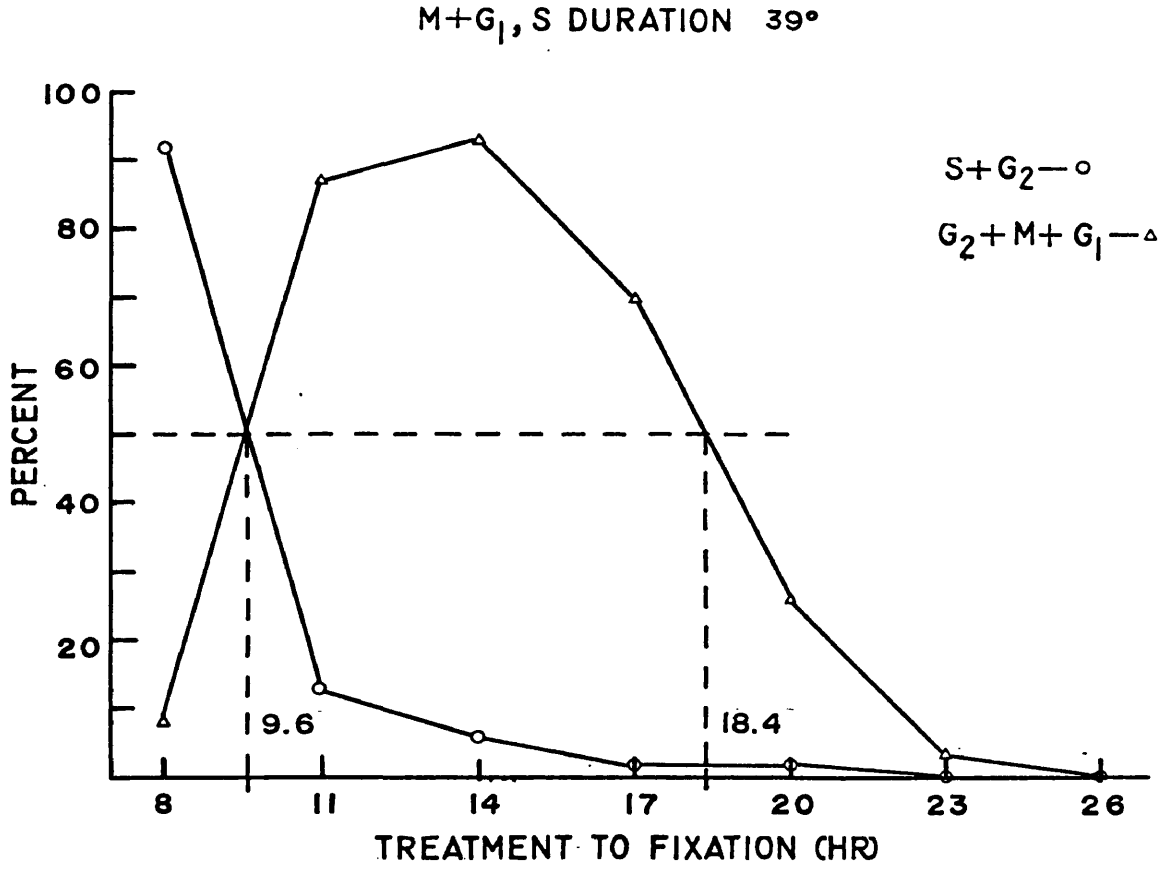
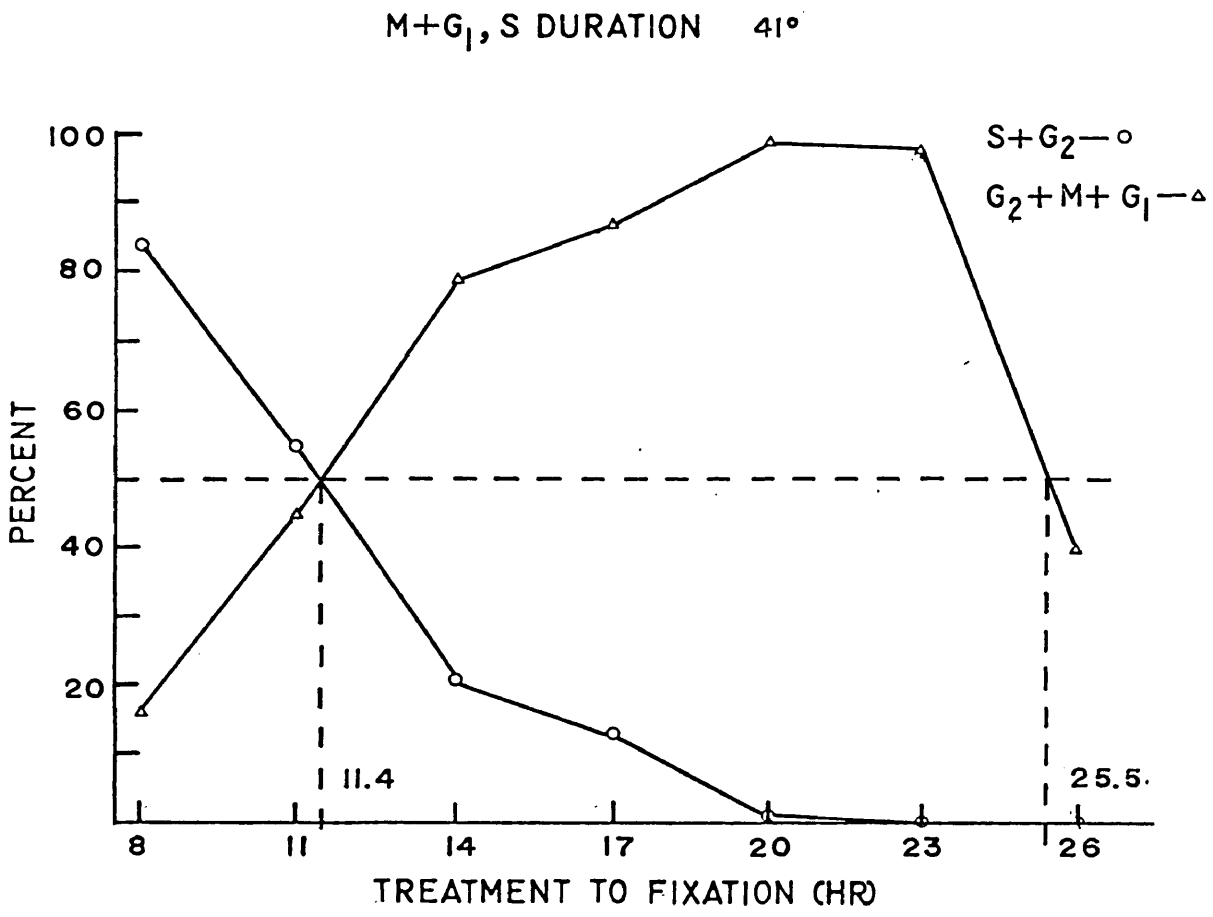

M+G1, S DURATION 39°
100
80
60
40
20
PERCENT
S+G2—o
G2+M+G1—Δ
9.6
18.4
8
11
14
17
20
23
26
TREATMENT TO FIXATION (HR)

Fig. 8. Experiment 5 results, 41°C.


M+G1, S DURATION 41°
S+G2—o
G2+M+G1—Δ
PERCENT
100
80
60
40
20
11.4
25.5
8
11
14
17
20
23
26
TREATMENT TO FIXATION (HR)

DISCUSSION

In this study, peak growth rate, in terms of overall *in vitro* lymphocyte growth kinetics, was observed in cultures incubated at 39°C. Lymphocytes in Experiment 1 underwent, on the average, more divisions when incubated at 39°C than at the other experimental temperatures. Therefore, incubation at 39°C provides the optimal thermal environment for overall proliferative growth of human lymphocytes *in vitro*. Incubation at temperatures above and below 39°C resulted in sharp decreases in the observed average number of divisions.

Suboptimal temperatures allowed a low rate of division in previously transformed cells. Conceivably, division would still be possible at temperatures even lower than those employed in this study. The slower growth observed at supraoptimal temperatures might have resulted from heat damage to the microtubules of the spindle apparatus and by interference with the separation of sister chromatids (Zeuthen, 1972). Incubation of mammalian cells at 43°-48°C will denature some cellular proteins and will induce structural damage of entire organelles (Landry and Marceau, 1978; 1979).

Although there was a sharp peak GRI at 39°C in Experiment 1, there was no clear peak in Experiment 3. Instead, GRI values at 37° and 39°C were essentially similar. This similarity indicates that the total duration of the division cycle at 39° is not different than that at 37°C. The difference in temperature optima noted between the two experiments probably results from a thermal differential in transfor-

mation rate. As noted previously, Ashman and Nahmias (1978) concluded that Con A-mediated transformation rates are not temperature-dependent. However, over an 18 hr period, PHA-stimulated lymphocytes incubated at 39°C exhibited an accelerated rate of entry into the division cycle based on the percentage of cells labeled with ^{3}H-TdR as compared with lymphocytes cultured at 37°, 35°, or 41°C. Apparently, cells which enter the division cycle earlier than others with the same proliferation rate will undergo more divisions than will later entries, assuming continued progress through successive divisions.

At temperatures above and below the 37°-39°C range in Experiment 3, GRI values indicated a lengthening of the cell cycle. Therefore, the temperature range 37°-39°C represents a broad optimum with respect to total division cycle length. However, when the overall kinetics of in vitro lymphocyte proliferative growth are considered, the optimal temperature is 39°C. At supra- and suboptimal temperatures, both decreased transformation rates and lengthened division cycle times contribute to the lower average number of divisions observed in cultures from Experiment 1.

As previously noted, there is some uncertainty as to the degree of thermosensitivity of each phase of the division cycle. The phases of the division cycle are delimited by the initiation and completion of DNA synthesis and mitosis. Initiation of both has been found to be under positive control mediated by cytoplasmic factors, thought to be proteins; completion of both is the result of an orchestrated series of events and processes, apparently independent of the aforementioned cytoplasmic factors (Thompson and McCarthy, 1968; Rao and Johnson, 1970; Kumar and Friedman, 1972; Graves, 1972).

The implication of this type of cytoplasmic cycle control is that incubation temperature may affect the duration of the separate phases in one of two ways: by influencing the production and/or activity of the factors which signal the initiation of S phase and mitosis or by influencing the actual progress of the phase, perhaps via a direct effect on reaction kinetics. Duration of G_1 would be influenced by a change in production and/or activity of the cytoplasmic factors. Enzymatic reactions occurring in G_1 would be influenced by temperature, due to thermal effects on reaction kinetics. However, the duration of G_1 is defined by the initiation of DNA synthesis and the effect temperature may have on the initiation of DNA synthesis may be thought of as rate-limiting with respect to the duration of G_1. The duration of G_2 would be affected similarly, although thermal influences on initiation of mitosis are rate-limiting. Durations of S phase and mitosis would, on the other hand, be controlled by the thermosensitivity of DNA synthesis and the division process, respectively. Both processes are certainly influenced by cytoplasmic signals which regulate the execution or precision of the process, but it seems likely that the rates of both processes would be limited by thermal influence on reaction kinetics.

If this type of temperature control is, indeed, in operation, then factor and process are influenced in different ways. Both $M+G_1$ and G_2 durations were shortest at 39°C although durations at 37°C were not dissimilar. Durations at 35° and 41°C were also similar but longer than those at 37° and 39°C. Therefore, it may be that factors which initiate DNA synthesis and mitosis are more efficiently produced and/or more active at 37°-39°C than at supra- and suboptimal temperatures. The activity of mitotic factors, extracted from mitotic HeLa cells have

been shown to be extremely heat labile (Sunkara, Wright, and Rao, 1979; Rao, Sunkara, and Wright, 1981). After incubation for only 10 min at 40°C, the factors lost 17% of their original activity; incubation at higher temperatures caused a loss of almost all of the original activity. However, incubation at 0°C only caused a slight decrease in activity. Mitotic factors from cells of species with core temperatures lower than those of mammals are also more heat labile; amphibian factors have been shown to lose most of their original activity when incubated at 37°C (Wasserman and Masui, 1976; Sunkara et al., 1979).

The cycle phases which, in this model, would be affected by temperature through direct influence of the process are S and mitosis. Apparently, the temporal progress of DNA synthesis is independent of cytoplasmic control, unlike G_1 or G_2 (Graves, 1972); temperatures will most likely directly affect the synthetic apparatus. In this study, as incubation temperature increased, so did observed S phase durations. The observed durations at 35° and 37°C were similar and shorter than those at 39° and 41°C which were similar to each other. It is possible that the lower incubation temperatures provide an optimal thermal environment for the progress of DNA synthesis. Although optimal for S phase duration, the lower incubation temperatures are not optimal for G_1 and G_2. This would suggest that temperature indeed affects factor and process in different ways. However, to ensure that human lymphocytes follow this pattern, this experiment should be repeated.

Although there was little discernable difference in division cycle durations in cells incubated at 37° and those at 39°C, the optimal incubation temperature in terms of overall lymphocyte <u>in vitro</u> growth kinetics appears to be 39°C. Traditionally, human lymphocyte cultures

have been maintained at 37°C. Future studies requiring high yields of mitotic cells from short-term cultures of human lymphocytes may be more profitably performed using 39° rather than 37°C as incubation temperature. However, it should be noted that since 39°C is higher than the normal core temperature in man, incubation of cells at this temperature may result in subtle biochemical changes in the cells. For this reason, quantitative or qualitative studies of the physiology of normal lymphocytes should employ the traditional incubation temperature of 37°C.

As mentioned previously, the normal core temperature in man is 37°C (Dinarello and Wolff, 1978). This temperature is maintained by the thermoregulatory center in the anterior hypothalamus, in a manner reminiscent of a thermostat (Kluger, 1978). The normal core temperature represents the "set-point" temperature for thermoregulation. Sensed internal temperatures above this "set-point" temperature invoke behavioral and physiological responses which cool the body. When core temperature falls below the "set-point", physiological and behavioral responses which lead to conservation of body heat are initiated. Infection of the body may result in a rise in core temperature by a process known as the febrile response, or fever (Feldberg, 1974; Dinarello and Wolff, 1978; Kluger, 1978). Fever is the result of an increase in the "set-point" (Cooper, Cranston, and Snell, 1964; Kluger, 1978), a condition qualitatively different from hyperthermia, where the "set-point" is normal, but core temperature is higher than normal (Snell and Atkins, 1968).

The immune system is intimately involved in the body's response to infection. It would therefore be reasonable to expect that increased core temperature due to a febrile response would influence the activity

and effectiveness of the immune system. Reptiles which are allowed to develop a fever in response to bacterial infection exhibit higher survival rates than did those which are not allowed to develop a fever (Kluger, Ringler, and Anver, 1975). Increased temperature due to a febrile response has also been shown to result in faster migration of leucocytes to the site of infection, although no differences in antibody levels were noted between reptiles which developed fevers and those which did not (Bernheim et al., 1977). It should be emphasized that in the studies mentioned above, temperature plays an indirect role in the body's fight against infection through the temperature-dependency of the immune system.

PHA, the mitogen used in this study, stimulates T lymphocytes specifically (Daguillard, 1973). In vivo, T lymphocytes function in the production of some lymphokines and in the process of cytotoxicity. These functions become manifest after antigenic stimulation causes transformation of the quiescent lymphocyte (Valdimarsson, 1975) in a manner similar to that occurring in vitro. By raising core temperature, fever may result in a higher rate of activation of immunologically active T lymphocytes and an earlier onset of lymphokine production and cell-mediated immune responses. Since there are no apparent temperature-dependent differences in division cycle durations at 37° and 39°C in vitro, it may be that there is little, if any, difference in the level of lymphokine production and/or cytotoxic activity once the lymphocytes have left G_0. The advantage afforded by fever would result from a higher activation rate, resulting in a swifter buildup of immunocompetent cells.

APPENDIX

APPENDIX A

TABLES

TABLE 1. THE DURATIONS OF $M+G_1$, S, AND G_2 AT INCUBATION TEMPERATURES OF 35°-41°C.

TEMPERATURE (°C)	$M+G_1$	S	G_2
35	11.6 hr (14.1)	5.1 hr (4.6)	2.5 hr
37	7.4 hr (9.5)	6.0 hr (5.5)	2.1 hr
39	7.2 hr (8.8)	8.0 hr (7.5)	1.6 hr
41	11.1 hr (14.1)	8.4 hr (7.9)	3.0 hr

TABLE 2. EXPERIMENT 1 - OVERALL GROWTH KINETICS

TEMPERATURE (°C)	M1	M2	M3	M4	M5
31	0	0	0	0	0
33	96	3	1	0	0
35	23	69	8	0	0
37	12	29	46	13	0
39	2	12	33	44	9
41	44	49	7	0	0
43	0	0	0	0	0

The headings "M1", "M2", etc., refer to the metaphase generation number, the number of divisions undergone by a cell in the presence of BUdR. "M1" signifies one division in BUdR, "M2" signifies two divisions, etc.

TABLE 3. EXPERIMENT 2 - THE TIME-COURSE OF PHA TRANSFORMATION

INCUBATION TIME (HR)	35°C	37°C	39°C	41°C
18	0.00%	0.00%	0.20%	0.10%
21	0.20	0.20	1.00	0.10
24	0.50	0.90	3.60	0.20
27	0.90	5.40	11.50	0.40
30	2.30	10.50	18.60	0.60
33	4.50	11.80	21.50	4.00

Percentages given are calculated from the number of labeled cells in a sample of 1000 cells from each culture.

TABLE 4. EXPERIMENT 3 - THE TIME-COURSE OF CELL DIVISION FOLLOWING TRANSFORMATION

METAPHASE GENERATION	31°C	33°C	35°C	37°C	39°C	41°C	43°C
M0.5	0	0	0	2	1	0	0
M1	55	14	13	5	4	31	0
M1.5	6	1	1	6	4	7	0
M2	39	61	45	20	14	45	0
M2.5	0	3	6	6	18	17	0
M3	0	16	33	27	18	0	0
M3.5	0	0	2	6	12	0	0
M4	0	0	0	17	19	0	0
M4.5	0	0	0	2	4	0	0
M5	0	0	0	9	5	0	0

Headings given as "M0.5" or "M1.5" refer to cells which exhibited replication bands; i.e., were in S phase at the time of addition of BUdR. A cell designated as "M1.5" underwent one complete S phase and part of a previous S phase while incubated with BUdR. The partial S phase is the phase the cell was in when BUdR was added to the culture.

TABLE 5. EXPERIMENT 4 - THE DURATION OF G_2

TREATMENT TO FIXATION (HR)	35°C	37°C	39°C	41°C
7	100	100	100	86
6	97	100	100	79
5	87	100	100	79
4	74	96	100	67
3	44	86	97	44
2	42	46	74	33
1	1	3	40	0

The results are given as the number of cells scored as being in S phase when BUdR was added at the indicated time.

TABLE 6. EXPERIMENT 5 - THE DURATIONS OF M+G_1 AND OF S; 35°C

TREATMENT TO FIXATION (HR)	M0.5	M1	M1.5	M2
6	100	0	0	0
8	37	63	0	0
11	4	96	0	0
14	2	98	0	0
17	1	99	0	0
20	0	89	10	1
23	1	21	33	45
26	0	6	25	68

TABLE 7. EXPERIMENT 5 - THE DURATIONS OF M+G_1 AND OF S; 37°C

TREATMENT TO FIXATION (HR)	M0.5	M1	M1.5	M2	M2.5
6	100	0	0	0	0
8	52	48	0	0	0
11	12	88	0	0	0
14	7	86	7	0	0
17	4	59	36	1	0
20	3	12	71	14	0
23	5	9	56	29	1
26	5	5	17	64	9

TABLE 8. EXPERIMENT 5 - THE DURATIONS OF M+G_1 AND OF S; 39°C

TREATMENT TO FIXATION (HR)	M0.5	M1	M1.5	M2	M2.5
6	100	0	0	0	0
8	92	8	0	0	0
11	13	87	0	0	0
14	6	93	1	0	0
17	2	70	28	0	0
20	2	26	71	1	0
23	0	3	29	68	0
26	0	0	6	92	2

TABLE 9. EXPERIMENT 5 - THE DURATIONS OF M+G_1 AND OF S; 41°C

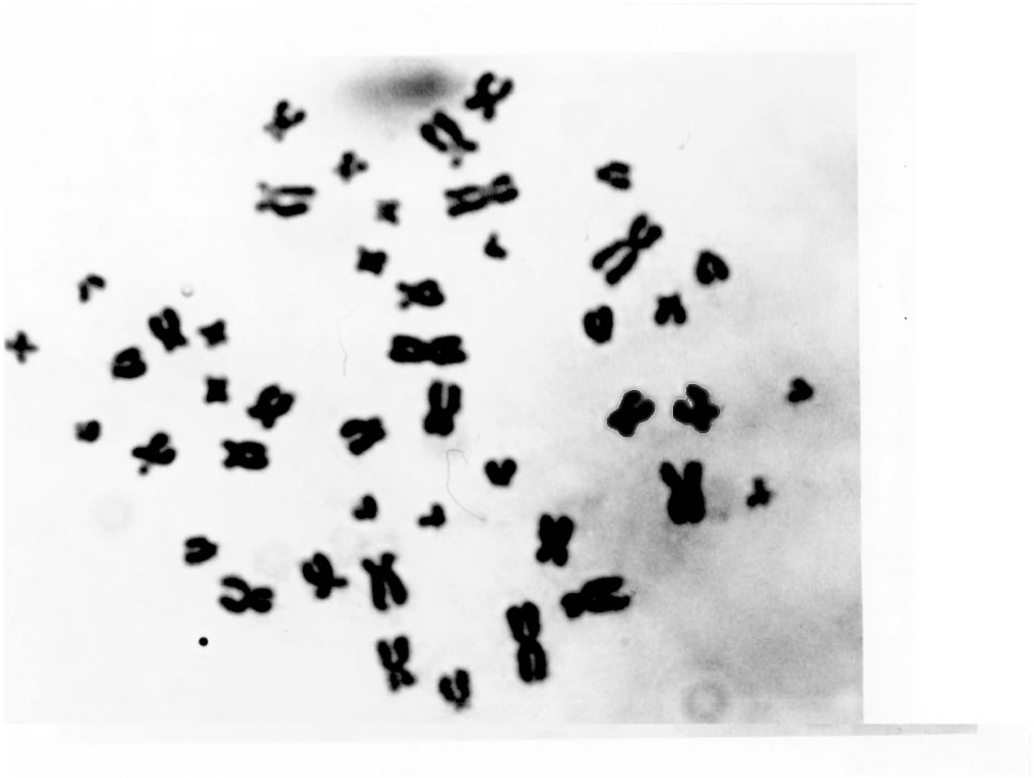
TREATMENT TO FIXATION (HR)	M0.5	M1	M1.5
6	99	1	0
8	84	16	0
11	55	45	0
14	21	79	0
17	13	87	0
20	1	99	0
23	0	98	2
26	0	98	2

APPENDIX B

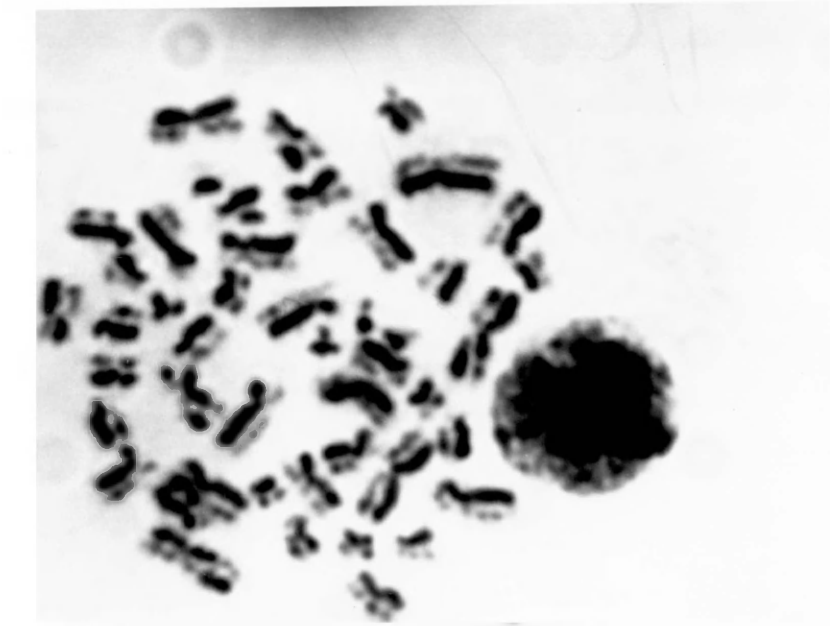
SISTER CHROMATID DIFFERENTIATION OF BUdR-SUBSTITUTED CHROMOSOMES

This spread exhibits replication bands. This cell began to utilize BUdR while in the final S phase prior to termination. The dark bands are those regions which had completed DNA synthesis before BUdR addition; lighter areas are those which are BUdR-labeled.

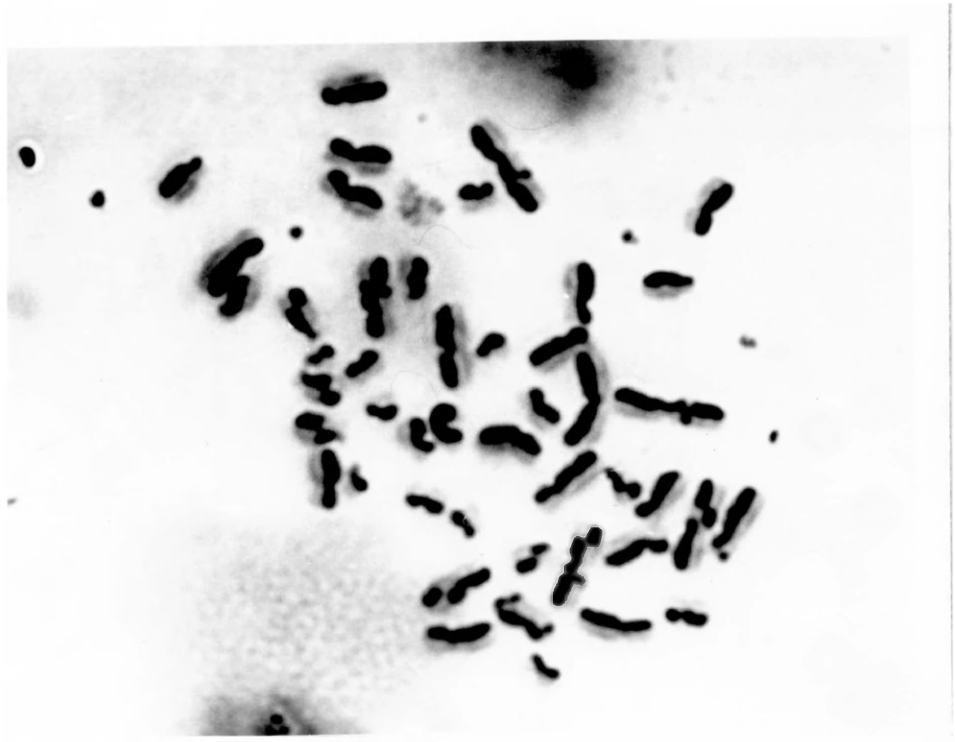
These chromosomes are uniformly stained; i.e., they are unifilarly substituted.



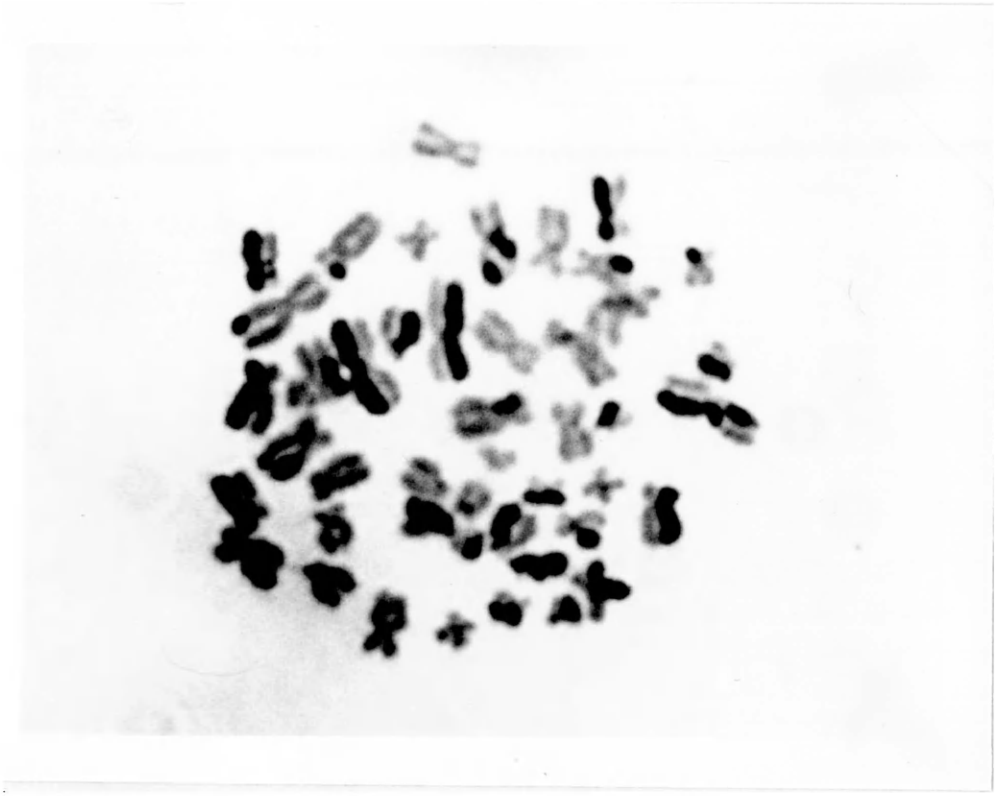
This metaphase spread is from a cell which began uptake of BUdR during its next-to-last S phase prior to termination. Replication bands are visible on the lighter chromatids. The darkly stained chromatids are unifilarly substituted.



This cell underwent two complete S phases in the presence of BUdR. In each chromosome, the darkly stained chromatid is unifilarly substituted, the lightly stained chromatid is bifilarly substituted. In several chromosomes, sites of sister chromatid exchange are readily apparent as isolocus switches of label between sister chromatids.



This cell underwent three complete S phases in the presence of BUdR. Cells which underwent more divisions exhibit progressively fewer darkly stained chromatids.



LITERATURE CITED

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Abercrombie, M., and J. E. M. Heaysman. 1954. Observations on the social behaviour of cells in tissue culture. II. "Monolayering" of fibroblasts. Exp. Cell Res., 6:293-306.

Ashman, R. B., J. N. Gomez-Barreto, and A. J. Nahmias. 1976. The effect of temperature on the in vitro transformation of human peripheral blood lymphocytes. Fed. Proc., 35:821.

Ashman, R. B., and A. J. Nahmias. 1977. Enhancement of human lymphocyte responses to phytomitogens in vitro by incubation at elevated temperatures. Clin. Exp. Immunol., 29:464-467.

Ashman, R. B., and A. J. Nahmias. 1978. Effect of incubation temperature on mitogen responses of lymphocytes from adult peripheral blood and from cord blood. Clin. Exp. Immunol., 33:319-326.

Bernheim, H. A., P. Bodel, P. Askenase, and E. Atkins. 1977. Mechanisms for life-saving effect of fever in infected lizards. Clin. Res., 25:A372.

Bloom, W., and D. W. Fawcett. 1975. A Textbook of Histology. W. B. Saunders Co., Philadelphia, p. 832.

Brucher, J., M. Teodorescu, A. Gaspar, D. T. Stefanescu, and S Bodulescu. 1973. Different optimal temperatures for the biologic activity of rabbit and human lymphocytes in vitro. Arch. Roum. Pathol. Exp. Microbiol., 32:297-300.

Carrel, A. 1912. On the permanent life of tissues outside of the organism. J. Exp. Med., 15:516-528.

Cooper, K. E., W. I. Cranston, and E. S. Snell. 1964. Temperature regulation during fever in man. Clin. Sci., 27:345-356.

Craig-Holmes, A. P., and M. W. Shaw. 1976. Cell cycle analysis in asynchronous cultures using the BUdR-Hoechst technique. Exp. Cell Res., 99:79-87.

Crossen, P. E., and W. F. Morgan. 1979. Proliferation of PHA-stimulated lymphocytes measured by combined autoradiography and sister chromatid differential staining. Exp. Cell Res., 118:423-427.

Daguillard, F. 1973. The differing responses of lymphocytes to mitogens, pages 571-583 in Proceedings of the Seventh Leucocyte Culture Conference. Academic Press, New York.

Defendi, V., and L. A. Manson. 1963. Analysis of the life-cycle in mammalian cells. Nature, 198:359-361.

Dinarello, C. A., and S. M. Wolff. 1978. Pathogenesis of fever in man. New Eng. J. Med., 298:607-612.

Eagle, H. 1955. Nutrition needs of mammalian cells in tissue culture. Science, 122:501-504.

Eagle, H. 1974. Some effects of environmental pH on cellular metabolism and function, pages 1-11 in B. Clarkson, and R. Baserga, Eds., Control of Proliferation in Animal Cells. Cold Spring Harbor Laboratory.

Eagle, H., and K. Piez. 1962. The population-dependent requirement by cultured mammalian cells for metabolites which they can synthesize. J. Exp. Med., 116:29-43.

Eddy, S., and J. C. Underhill. 1971. How to Know the Freshwater Fishes. Wm. C. Brown Co., Dubuque, Iowa, p. 43.

Eisinger, M., J. S. Lee, J. M. Hefton, Z. Darzynkiewicz, J. W. Chiao, and E. de Harven. 1979. Human epidermal cell cultures: Growth and differentiation in the absence of dermal components or medium supplements. Proc. Nat. Acad. Sci., 76:5340-5344.

Feldberg, W. 1975. Body temperature and fever: changes in our views during the last decade. Proc. R. Soc. Lond. B., 191:199-229.

Fusening, N., and P. K. M. Worst. 1975. Mouse epidermal cell cultures. Exp. Cell Res., 93:443-457.

Graves, J. A. M. 1972. DNA synthesis in heterokaryons formed by fusion of mammalian cells from different species. Exp. Cell Res., 72:393-403.

Grzeschik, K. H., M. A. Kim, and R. Johannsmann. 1975. Late replicating bands of human chromosomes demonstrated by fluorochrome and Giemsa staining. Humangenetik, 29:41-59.

Heddle, J. A., S. Wolff, D. Whissell, and J. E. Cleaver. 1967. Distribution of chromatids at mitosis. Science, 158:929-931.

Hickman, C. P., C. P. Hickman, Jr., F. M. Hickman, and L. S. Roberts. 1979. Integrated Principles of Zoology. The C. V. Mosby Co., St. Louis, p. 802.

Hoo, J. J., and M. I. Parslow. 1979. Relation between the SCE points and the DNA replication bands. Chromosoma, 73:67-74.

Kato, H. 1974. Spontaneous sister chromatid exchanges detected by a BUdR-labelling method. Nature, 251:70-72.

Kluger, M. J. 1978. The evolution and adaptive value of fever. Am. Sci., 66:38-43.

Kluger, M. J., D. H. Ringler, and M. R. Anver. 1975. Fever and survival. Science, 188:166-168.

Kodama, S. 1930. Physiological studies on tissue in vitro. I. Influence of temperature upon the growth of fibroblasts in coverglass cultures. Cytologia, 2:77-80.

Kumar, K. V., and D. L. Friedman. 1972. Initiation of DNA synthesis in HeLa cell-free system. Nat. New Biol., 239:74-76.

Landry, J., and N. Marceau. 1978. Rate-limiting events in hyperthermic cell killing. Radiat. Res., 74:573-585.

Landry, J., and N. Marceau. 1979. Cell growth recovery after treatments at various supraoptimal temperatures. Cancer Res., 39: 1218-1223.

Latt, S. A. 1973. Microfluorometric detection of deoxyribonucleic acid replication in human metaphase chromosomes. Proc. Nat. Acad. Sci., 70:3395-3399.

Marcelo, C. L., Y. G. Kim, J. L. Kaine, and J. J. Voorhees. 1978. Stratification, specialization, and proliferation of primary keratinocyte cultures. Evidence of a functioning in vitro epidermal cell system. J. Cell Biol., 79:356-370.

Naspitz, C. K., and M. Richter. 1968. The action of phytohemagglutinin in vivo and in vitro, a review. Prog. Allergy, 12:1-85.

Perry, P., and S. Wolff. 1974. New Giemsa method for the differential staining of sister chromatids. Nature, 251:156-158.

Plumb, J. A., and K. Wolf. 1971. Fish cell growth rates. Quantitative comparison of RTG-2 cell growth at 5-25°C. In Vitro, 7: 42-45.

Rao, P. N., and J. Engelberg. 1965. HeLa cells: Effects of temperature on the life cycle. Science, 148:1092-1094.

Rao, P. N., and R. T. Johnson. 1970. Mammalian cell fusion: Studies on the regulation of DNA synthesis and mitosis. Nature, 225: 159-164.

Rao, P. N., P. S. Sunkara, and D. A. Wright. 1981. Chromosome condensation factors of mammalian cells, pages 49-60 in F. E. Arrighi, P. N. Rao, and E. Stubblefield, Eds., Genes, Chromosomes, and Neoplasia. Raven Press, New York.

Richardson, K. C., L. Jarett, and E. H. Finke. 1960. Embedding in epoxy resins for ultrathin sectioning in electron microscopy. Stain Tech., 35:313-323.

Rothman, S. 1954. Physiology and Biochemistry of the Skin. The University of Chicago Press, Chicago, p. 253.

Sanford, K. K., W. R. Earle, and G. D. Likely. 1948. The growth in vitro of single isolated tissue cells. J. Nat. Cancer Inst., 9: 229-246.

Schmidt-Nielsen, K. 1964. Animal Physiology. Prentice-Hall, Inc., Englewood Cliffs, p. 41.

Schneider, L. K., and H. D. Goldman. 1974. Effects of incubation temperature on the kinetics of cultured lymphocytes from the opossum Didelphis virginiana. Experientia, 30:1192-1193.

Sisken, J. E. 1963. Analysis of variations in intermitotic time, page 143 in G. C. Rose, Ed., Cinemicrography in Cell Biology. Academic Press, New York.

Sisken, J. E., L. Morasca, and S. Kibby. 1965. Effects of temperature on the kinetics of the mitotic cycle of mammalian cells in culture. Exp. Cell Res., 39:103-116.

Snell, E. S., and E. Atkins. 1968. The mechanisms of fever, pages 397-419 in E. E. Bittar and N. Bittar, Eds., The Biological Basis of Medicine, v. 2. Academic Press, New York.

Sunkara, P. S., D. A. Wright, and P. N. Rao. 1979. Mitotic factors from mammalian cells: A preliminary characterization. J. Supramol. Struct., 11:189-195.

Thestrup-Pedersen, K. 1972. Temperature optimum and rabbit blood cell culture. Acta. Pathol. Microbiol. Scand. B, 80:775-776.

Thompson, L. R., and B. J. McCarthy. 1968. Stimulation of nuclear DNA and RNA synthesis by cytoplasmic extracts in vitro. Biochem. Biophys. Res. Com., 30:166-172.

Tice, R., E. L. Schneider, and J. M. Rary. 1976. The utilization of bromodeoxyuridine incorporation into DNA for the analysis of cellular kinetics. Exp. Cell Res., 102:232-236.

Todaro, G. J., G. K. Lazar, and H. Green. 1965. The initiation of cell division in a contact-inhibited mammalian cell line. J. Cell. Comp. Physiol., 66:325-333.

Valdimarsson, H. 1975. Effector mechanisms in cellular immunity, pages 179-196 in M. J. Hobart, and I. M. McConnell, Eds., The Immune System. Blackwell Scientific Publications, Oxford.

Walters, R. A., R. A. Tobey, and R. L. Ratliff. 1973. Cell-cycle-dependent variations of deoxyribonucleoside triphosphate pools in Chinese hamster cells. Biochim. Biophys. Acta., 319:336-347.

Wasserman, W. J., and Y. Masui. 1976. A cytoplasmic factor promoting maturation: Its extraction and preliminary characterization. Science, 191:1266-1268.

Watanabe, I., and S. Okada, 1967. Effects of temperature on growth rate of cultured mammalian cells (L5178Y). J. Cell Biol., 37: 309-323.

Webber, L. M., J. M. Brasch, and D. R. Smyth. 1981. DNA extraction during Giemsa differentiation of chromatids singly and doubly substituted with BrdU. Chromosoma, 81:691-700.

Weber, K. E. 1979. Cell Phases During Endoreduplication Induced by Colcemid or Radiation in Cultured Human Lymphocytes. M. A. Thesis. College of William and Mary.

Zakharov, A. F., and N. A. Egolina. 1972. Differential spiralization along mammalian mitotic chromosomes I. BUdR-revealed differentiation in Chinese hamster chromosomes. Chromosoma, 38:341-365.

Zeuthen, E. 1972. Inhibition of chromosome separation in cleaving Psammechinus eggs by elevated temperature. Exp. Cell Res., 72: 337-344.

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